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Ibogaine alters synaptosomal and glial glutamate release and uptake

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Ibogaine has aroused expectations as a potentially innovative medication for drug addiction. It has been proposed that antagonism of the NMDA receptor by ibogaine may be one of the mechanisms underlying its antiaddictive properties; glutamate has also been implicated in ibogaine-induced neurotoxicity. We here report the effects of ibogaine on [³H]glutamate release and uptake in cortical and cerebellar synaptosomes, as well as in cortical astrocyte cultures, from mice and rats. Ibogaine (2–1000 μ M) had no effects on glutamate uptake or

release by rat synaptosomes. However, ibogaine (500–1000 μ M) significantly inhibited the glutamate uptake and stimulated the release of glutamate by cortical (but not cerebellar) synaptosomes of mice. In addition, ibogaine (1000 μ M) nearly abolished glutamate uptake by cortical astrocyte cultures from rats and mice. The data provide direct evidence of glutamate involvement in ibogaine-induced neurotoxicity. *NeuroReport* 12:263–267 © 2001 Lippincott Williams & Wilkins.

Key words: Astrocyte; Glutamate; Ibogaine; Mice; Rats; Release; Synaptosomes; Uptake

INTRODUCTION

Glutamate, the main excitatory neurotransmitter in the mammalian brain, is believed to play important roles in several physiological and pathological processes. Glutamatergic neurotransmission is achieved through ionotropic (ligand-gated ion channel) and metabotropic (coupled to cellular effectors) receptors [1]. Specifically, the NMDA ionotropic glutamate receptor subtype seems to be crucial in plastic changes associated with normal brain function [1]. However, over-stimulation of the glutamatergic system has been implicated in acute neurological disorders as well as in neurodegenerative diseases [2]. Glutamate concentrations are adequately maintained (below toxic levels) in the synaptic cleft by means of neuronal and, especially, by glial uptake. Glial uptake is performed by two carrier proteins located in astrocytes (glutamate transporter 1, GLT1; and glutamate/aspartate transporter, GLAST) [3,4].

Ibogaine, an indole alkaloid, has appeared in drug addiction therapy scenario as a truly innovative drug, arousing high expectations [5,6]. Ibogaine has been shown to interfere with various neurotransmitter systems [7], including the glutamatergic, acting as a non-competitive NMDA antagonist [6,8]. Because the glutamatergic system has also been implicated in drug addiction [9], this property may be relevant to the putative ibogaine antiaddictive properties. In fact, it has been shown that NMDA antagonists attenuate or reverse the development of toler-

ance, dependence and/or sensitization induced by several drugs of abuse [9–11].

Despite relevant evidence of ibogaine antiaddictive properties, obtained from *in vivo* and *in vitro* animal data as well as clinical accounts [5,6,8,12], the development of ibogaine as a treatment for drug addiction has been halted by data suggesting serious neurotoxicity [13–16]. It has been suggested that glutamate may play a central role in ibogaine-induced neurotoxicity. Specifically, it was postulated that ibogaine activation of inferior olivary neurons leads to excessive release of glutamate at climbing fibers, resulting in Purkinje cells loss [14]. However, there is no direct evidence that ibogaine could lead to over-stimulation of the glutamatergic system.

Considering the presumed importance of glutamate in mediating both the antiaddictive and neurotoxic properties of ibogaine, the purpose of this study was to investigate the effects of ibogaine on glutamate release and uptake by cortical and cerebellar synaptosomes prepared from mouse and rat brain. In addition, we have studied the effects of ibogaine on glutamate uptake by cortical astrocyte cultures from mice and rats.

MATERIALS AND METHODS

Animals: Male albino adult mice (CF-1 strain), bred at the Instituto de Pesquisas Biológicas (Porto Alegre, Brazil), and Wistar rats from our own breeding colony were used. The

animals were kept in separate animal rooms, on a 12:12 h light:dark cycle, at a room temperature of 22°C, with free access to food and water.

Preparation of synaptosomes: Animals were decapitated and the cortices and cerebella were removed and used to prepare synaptosomes on a discontinuous Percoll gradient according to Dunkley *et al.* [17]. Protein concentration was measured according to the method of Lowry *et al.* [18].

[³H]Glutamate release: Determination of [³H]glutamate release was accomplished according to the method described by Migués *et al.* [19]. The synaptosomal preparation was incubated in Hepes buffered salt solution (HBSS, composition in mM: HEPES 27, NaCl 133, KCl 2.4, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 12, CaCl₂ 1.0), pH 7.4 (adjusted with HCl), for 15 min at 37°C in the presence of [³H]glutamate (Amersham, sp. act. 53 Ci/mmol, final concentration 5×10^{-7} M). Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at $16\,000 \times g$ for 1 min. Supernatants were discarded, and the pellets were washed four times in HBSS by centrifugation at $16\,000 \times g$ for 1 min at 4°C. To assess the basal release of [³H]glutamate, the final pellet was resuspended in HBSS and incubated for 60 s in the absence (control) or presence of ibogaine hydrochloride (2–1000 μ M) at 37°C. Incubation was terminated by immediate centrifugation ($16\,000 \times g$, 1 min). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. The released [³H]glutamate was calculated as a percentage of the total amount of radiolabel present in the synaptosomes at the start of the incubation period. K⁺-stimulated [³H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization. The effect of ibogaine (1 mM) on [³H]glutamate release by cortical synaptosomes in the absence of Ca²⁺ was performed in a medium similar to that described above, except that CaCl₂ was omitted and 2 mM EGTA were added. In some experiments tetrodotoxin (TTX; 5 μ M) was added with ibogaine (1 mM) and veratridine (20 μ M).

[³H]Glutamate Uptake by synaptosomes: The synaptosomal preparation was washed twice by suspending in 3 vol 0.3 M sucrose, in 15 mM Tris/acetate buffer (pH 7.4) and centrifuging at $35\,000 \times g$ for 15 min. The final pellet was resuspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in HBSS (composition in mM: HEPES 24, NaCl 119, KCl 2.1, MgSO₄ 1.08, KH₂PO₄ 1.08, glucose 10.8, CaCl₂ 0.9), pH 7.4 (adjusted with HCl), in the presence of [³H]glutamate (final concentration 100 nM) in the absence or in the presence of ibogaine hydrochloride (2–1000 μ M), for 1 min at 37°C. The reaction was stopped by filtration through GF/B filters. The filters were washed three times with 3 ml ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The radioactivity retained on the filters was measured in a Wallac scintillation counter. Specific [³H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which choline chloride was substituted for NaCl.

Primary cortical astrocyte cultures: The method of Swanson *et al.* [20] for preparing primary cortical astrocyte cultures was used with minor modifications, as described below. The cortical tissue of newborn mice or rats was dissociated mechanically. The dissociated cells were washed, suspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and plated in Falcon 24 well tissue culture plates at an approximate density of 5×10^4 cells/cm². The cultures were maintained in a humidified, 5% CO₂ incubator at 37°C. The medium was exchanged weekly. At confluence (days 13–15), 10 μ M cytosine arabinoside was added and after 48 h this medium was replaced with medium containing 3% FBS and 0.15 mM di-butylcAMP (dBcAMP). Addition of dBcAMP to the media induced the expression of GLT-1 and increased the expression of GLAST [20]. Cultures were used at 25–30 days *in vitro*.

[³H]Glutamate uptake in astrocyte cultures: Glutamate uptake was measured as described by Swanson *et al.* [20]. Briefly, the culture media were replaced with a modified HBSS containing 2 mM glucose buffered to pH 7.2 with 5 mM piperazine-N,N'-bis[ethanesulfonic acid] (PIPES), and pre-incubated for 30 min at 37°C, in the absence or in the presence of ibogaine (0.1 or 1 mM). After this preincubation period uptake was started by adding 0.01 μ Ci/ml L-[³H]glutamate (Amersham, sp. act. 53 Ci/mmol) plus 100 μ M unlabeled glutamate to each culture well. The uptake was terminated after 7 min of incubation (37°C), by washing the cell cultures twice with ice-cold HBSS, followed immediately by cell lysis in 0.5 N NaOH/0.05% lauryl sulfate. Non-specific uptake was measured in HBSS containing choline chloride instead of NaCl. Aliquots were taken for scintillation counting and protein assays [18].

Measurement of lactate dehydrogenase (LDH) activity: In order to evaluate the integrity of the synaptosomes and astrocyte cultures after the incubation period, an aliquot of the supernatant was withdrawn and frozen for later determination of LDH activity. LDH activity was measured using an assay kit (Doles Reagents, Brazil) modified as described by Romano *et al.* [21]. LDH activity was assessed by measuring the amount of a colored complex derived from the NADH formed by the enzymatic reaction using a spectrophotometric method (510 nm).

Statistical analysis: Statistical significance was assessed by ANOVA, followed by Duncan's test when appropriate. A value of $p < 0.05$ was considered to be significant, and F values are presented only when a significant difference was found.

RESULTS

Figure 1 shows the effects of ibogaine on [³H]glutamate release from mice synaptosomes. Ibogaine (500 and 1000 μ M) significantly increased basal ($F(5,29) = 2.54$, $p < 0.05$) but not K⁺-stimulated glutamate release by cortical synaptosomes (Fig. 1a). In cerebellar synaptosomes (Fig. 1b) ibogaine (up to 1 mM) did not modify basal or K⁺-stimulated [³H]glutamate release.

In order to determine the Ca²⁺ dependence of the effect of ibogaine on glutamate release, mice cortical synapto-

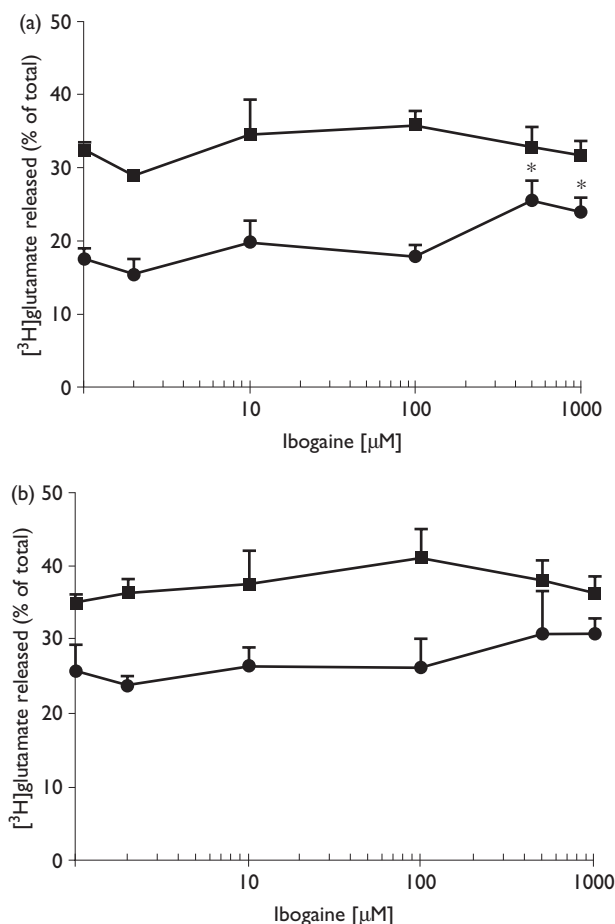


Fig. 1. Effects of ibogaine on basal (circles) and K^+ -stimulated (squares) release of $[^3\text{H}]$ glutamate from mice cortical (a) and cerebellar (b) synaptosomes. Glutamate released is expressed as percentage of total radioactivity content. Data are means $\pm$ s.e.m. from 4–8 independent experiments. * $p < 0.05$, compared with control (basal release in the absence of ibogaine), ANOVA/Duncan.

somes were incubated in the absence of Ca^{2+} and in the presence of 2 mM EGTA (Fig. 2). The effect of the absence of Ca^{2+} on ibogaine-induced glutamate release was compared with the effect of the absence of Ca^{2+} on K^+ -stimulated glutamate release. Two-way ANOVA (2 calcium conditions $\times$ 3 treatments) revealed a significant effect of treatment ($F(2,36) = 26.50$, $p < 0.01$) and a significant calcium condition $\times$ treatment interaction ($F(2,36) = 3.90$, $p < 0.05$). *Post-hoc* analysis (Duncan's test) indicated that ibogaine-induced $[^3\text{H}]$ glutamate release was not modified by the absence of Ca^{2+} , while K^+ -stimulated $[^3\text{H}]$ glutamate release was significantly attenuated when Ca^{2+} was absent.

The role of Na^+ channels on ibogaine-induced glutamate release was investigated by adding the Na^+ channel blocker TTX (5 μM) to the incubation (Fig. 3). Two-way ANOVA (2 TTX conditions $\times$ 3 treatments) revealed a significant effect of treatment ($F(2,29) = 12.18$, $p < 0.01$) and a significant TTX condition $\times$ treatment interaction ($F(2,29) = 8.82$, $p < 0.01$). *Post-hoc* analysis revealed that TTX did not affect ibogaine-induced $[^3\text{H}]$ glutamate release, while completely

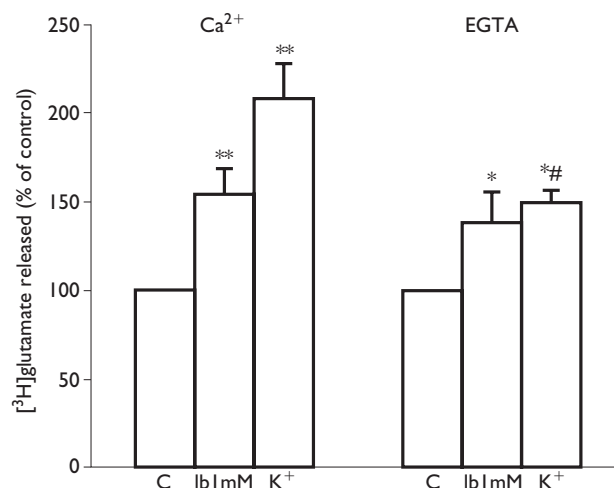


Fig. 2. Effect of the absence of calcium on $[^3\text{H}]$ glutamate release induced by ibogaine (Ib 1 mM) or 40 mM KCl (K^+) from mice cortical synaptosomes. Glutamate released is expressed as a percentage of control (low K^+ concentration, without ibogaine). Data are means $\pm$ s.e.m. from seven independent experiments. * $p < 0.05$ and ** $p < 0.01$, compared with respective control. # $p < 0.05$, compared with K^+ -induced release in the presence of Ca^{2+} , ANOVA/Duncan.

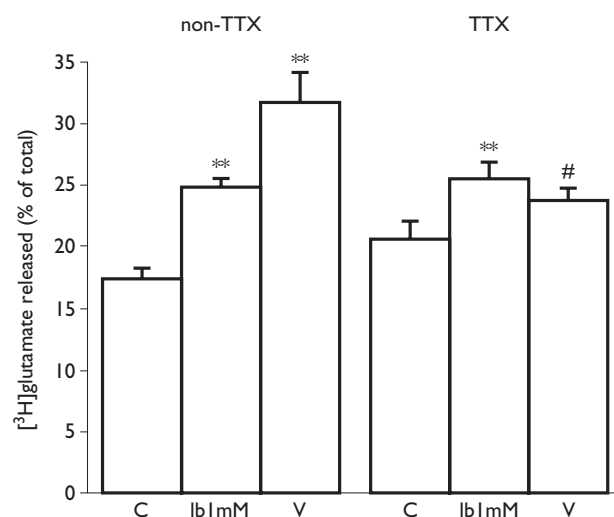


Fig. 3. Effect of TTX on $[^3\text{H}]$ glutamate release induced by ibogaine (Ib 1 mM) or 20 μM veratridine (V) from mice cortical synaptosomes. Glutamate released is expressed as percentage of total radioactivity content. Data are means $\pm$ s.e.m. from 4–6 independent experiments. ** $p < 0.01$, compared with respective control. # $p < 0.05$, compared with veratridine without TTX, ANOVA/Duncan.

abolishing $[^3\text{H}]$ glutamate release induced by veratridine (20 μM), a Na^+ channel activator.

In contrast with the results obtained in mice synaptosomes, ibogaine had no effect on $[^3\text{H}]$ glutamate release by cortical or cerebellar synaptosomes from rats (data not shown).

Figure 4 shows ibogaine effects on $[^3\text{H}]$ glutamate uptake. Ibogaine (500 and 1000 μM) significantly inhibited $[^3\text{H}]$ glutamate uptake (27% and 38%, respectively) by

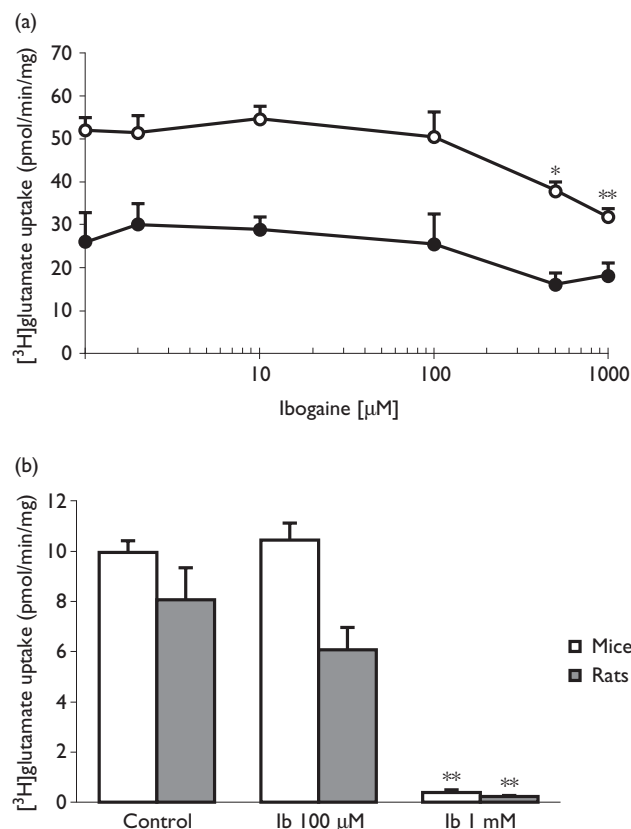


Fig. 4. Effects of ibogaine on [3 H] glutamate uptake by cortical (open circles) and cerebellar (filled circles) synaptosomes of mice (a), and by cortical astrocyte cultures of mice and rats (b). [3 H]Glutamate uptake is expressed as pmol/min/mg of protein. Data are means $\pm$ s.e.m. from 3–5 independent experiments. * $p < 0.05$; ** $p < 0.01$, compared with control, ANOVA/Duncan.

cortical mice synaptosomes ($F(5,20) = 7.64$, $p < 0.01$), while having no significant effect on the [3 H]glutamate uptake by mice cerebellar synaptosomes (Fig. 4a). Ibogaine had no effects on [3 H]glutamate uptake by cortical or cerebellar synaptosomes from rats (data not shown). Ibogaine 1 mM (but not 100 μ M) inhibited [3 H]glutamate uptake by cortical astrocyte cultures from mice (96%; $F(2,6) = 135.9$, $p < 0.01$) and rats (97%; $F(2,9) = 29.26$, $p < 0.01$; Fig. 4b).

Synaptosomes and astrocyte cultures did not show any significant leakage of the cytosolic marker LDH after incubation with ibogaine (2–1000 μ M; data not shown), indicating that ibogaine did not disrupt astrocyte cells or synaptosomal plasma membranes in our assay conditions.

DISCUSSION

The glutamatergic system is said to be implicated in drug addiction [9] and its over-stimulation is involved in neural damage [2]. Likewise, the putative antiaddictive and neurotoxic properties of ibogaine have been associated with glutamate [5,8,10,14]. In this study we evaluated effects of ibogaine on extracellular glutamate levels, by investigating its effects (2–1000 μ M) on [3 H] glutamate release and uptake by cortical and cerebellar synaptosomes of mice

and rats, and on [3 H]glutamate uptake by mice and rats cortical astrocyte cultures.

At concentrations likely to be present in the brain with therapeutically relevant ibogaine antiaddictive doses (4–133 μ M) [8,22–24] we did not find any significant effects on [3 H]glutamate release or uptake by synaptosomes or cortical astrocyte cultures of rats or mice.

The main findings of our study indicate that higher concentrations of ibogaine increased (37–45%) glutamate release and inhibited (27–38%) glutamate uptake in cortical synaptosomes of mice. Moreover, there was an almost complete inhibition of glutamate uptake by cortical astrocyte cultures in mice (96%) and rats (97%).

Considering the crucial role of astrocyte uptake in physiological and pathological processes [4], and that neuronal release and astrocytic uptake are concomitant processes in neural activity, the effect of ibogaine on astrocyte uptake seems to be the most relevant. The absence of a significant LDH leakage after synaptosomal or cell culture incubation with ibogaine indicate that increased glutamate release and glutamate uptake inhibition can not be attributed to the disruption of plasma membrane. Ibogaine-induced glutamate release was not observed in the presence of depolarizing K^+ concentrations. In addition, unlike K^+ -stimulated glutamate release, ibogaine-induced release was not affected by removing Ca^{2+} from the medium. These data indicate that unlike K^+ , ibogaine may induce synaptosomal depolarization through a mechanism(s) independent of exogenous Ca^{2+} . Nonetheless, our results did not rule out a possible involvement of intracellular calcium stores in ibogaine-induced glutamate release, which could be mobilized through the activation of some metabotropic receptor.

TTX, which inactivates voltage-sensitive Na^+ channels [25], did not alter ibogaine-induced [3 H]glutamate release, indicating that voltage-sensitive Na^+ channels are not involved in ibogaine-induced glutamate release.

Altogether, the results suggest that ibogaine could increase glutamate concentrations in the synaptic cleft through an stimulation of neuronal glutamate release, mediated by a synaptic depolarization independent of exogenous Ca^{2+} and of voltage sensitive Na^+ channels. It has been reported that ibogaine binds to sigma-2 receptors [26] and ibogaine activates sigma-2 receptors, suggesting that this could induce glutamate release, leading to neurotoxicity [5].

Extracellular glutamate concentrations are usually maintained at low levels by its Na^+ -dependent transport into neurons and, especially, astrocytes [3]. Ibogaine inhibited both glutamate uptake by mice cortical synaptosomes (neuronal transporters) and by mice cortical astrocyte cultures. These results reinforce the proposal that high ibogaine concentrations could increase extracellular glutamate, leading to excitotoxic concentrations in the synaptic cleft.

It is unlikely that the effects of ibogaine reported in the present study could be related to the antiaddictive property of this compound. The effects here reported were observed at ibogaine concentrations thought to be higher than those proposed to be therapeutically effective [8,22,23]. Moreover, therapeutic properties of ibogaine have been attributed to a depression and not to a stimulation of

glutamatergic activity [6,10]. Nevertheless, an ibogaine-induced increase of extracellular glutamate concentrations could be implicated in the neurotoxic effects of this compound. Indeed, it has been suggested that the Purkinje cell loss observed after *in vivo* administration of ibogaine to rats could be related to the activation of inferior olivary neurons, leading to excessive glutamate release at climbing fibers [14]. Our data are in agreement with this hypothesis, and we have provided for the first time direct evidence that ibogaine inhibits astrocytic uptake; this effect could amplify glutamate excitotoxicity brought about by glutamate release by olivary neurons.

Clinical data (including postmortem studies) suggest low risk for ibogaine-induced cerebellar toxicity in humans in the dose range purported to be effective for opiate and cocaine detoxification [6]. However, caution is necessary since ibogaine toxicity seems to be dependent on several factors, including sex, dosage regimen, and species [13]. Discrepancies in ibogaine effects between rats and mice have been reported previously [16], including differential effects in cortex. Accordingly, our data point to species and regional differences concerning the effects of ibogaine on glutamate release and uptake. The differential effects of ibogaine on glutamate uptake in rats and mice, astrocytes and neurons, cortex and cerebellum, could be related to differences in the expression of the various transport systems involved in glutamate uptake.

CONCLUSION

The data reported here contribute to the understanding of the mechanisms of actions of ibogaine, by demonstrating direct effects of ibogaine on glutamate uptake and release. We believe that these effects are unlikely to participate in

the purported therapeutic antiaddictive properties of this alkaloid, but may be relevant to its eventual toxicity.

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